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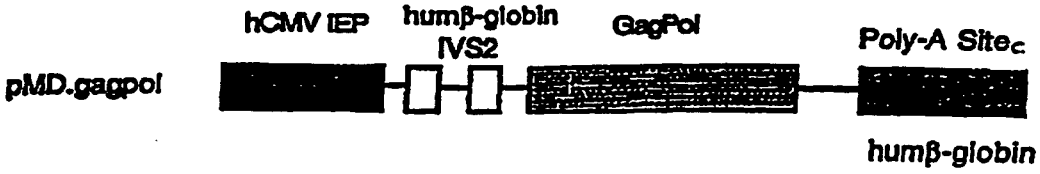

(57) Abstract

The present invention relates to a stable, pseudotyped retrovirus packaging cell line comprising packaging cells which generate helper-free recombinant pseudotyped retrovirus. The packaging cell line comprises one or more non-retroviral expression constructs, such as an expression construct with the human cytomegalovirus (CMV) immediate early promoter or derivatives of this promoter (e.g., pMD), which direct expression of: (a) retroviral *gagpol* genes and (b) a non-retroviral gene which is under the control of an inducible operator system and whose gene product pseudotypes retroviral core virions. The present invention further relates to a method of making a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus. The present invention further relates to the particular packaging cell lines described herein (i.e., H29 gagpol, H29 new gagpol) and the particular cells and constructs (i.e., packaging elements) used to produce the stable, pseudotyped retrovirus packaging cell line described herein (e.g., H29 cells and pMD, pMDtet, pMDtet.G, PMD.gagpol, pMD.new gagpol constructs). The present invention relates to a retroviral vector for producing a cDNA library for expression in mammalian cells, comprising two retroviral long terminal repeats, a cloning site for insertion of cDNA and a cytomegalovirus promoter. The invention also relates to a cDNA library for expression in mammalian cells, the library comprising retroviral vectors of the present invention. The present invention also relates to a method of expression cloning in mammalian cells. The present invention also relates to a method of cDNA expression cloning in mammalian cells. The present invention also relates to a method of identifying a gene defect responsible for a mutant phenotype using cDNA expression cloning by complementation in mammalian cells.

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STABLE PACKAGING CELL LINE PRODUCING PSEUDOTYPED RETROVIRUSES

Related Applications

This application is a Continuation-in-Part of U.S.S.N. 08/555,155, entitled "Stable Packaging Cell Line Producing Pseudotyped Retroviruses For Gene Transfer", by Daniel S. Ory, Michel Sadelain and Richard C. Mulligan, filed November 8, 1995 and of U.S.S.N. 08/651,050, entitled "A Method For Generation Of Retroviral cDNA Expression Libraries With A Vesicular Stomatitis Virus-G (VSV-G) Host Range For Expression Cloning By Complementation", by Daniel S. Ory and Jean E. Schaffer, filed May 21, 1996. The teachings of each are incorporated herein by reference in their entirety.

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Background

Recombinant retroviruses are useful for *in vivo* and *in vitro* gene expression and for production of proteins of interest in eukaryotic host cells. Generally, recombinant retroviruses are produced by introducing a suitable proviral DNA vector into mammalian cells that produce the necessary viral proteins for encapsidation of the desired recombinant RNA and generation of infectious recombinant virions. Since, for most gene transfer applications, the generation of pure stocks of recombinant virus free of replication-competent helper virus is desirable, there has

been considerable interest in developing cell lines that produce the necessary viral gene products for the generation of recombinant retrovirus, yet do not themselves either yield detectable helper virus or transfer viral genes (Coffin, J., *In:RNA Tumor Viruses,* Weiss, R. *et al.* (ed.). Cold Spring Harbor Laboratory, Vol. 2, pp.36-73 (1985); Mann, R. *et al., Cell, 33*:153-159 (1983); Watanabe, S., *et al., Mol. Cell Biol., 3*:2241-2249 (1983); Cone, R.D., *et al., PNAS, USA, 81*:6349-6353 (1984); Miller, A.D., *et al., Mol. Cell Biol., 6*:2895-2902 (1986); Bosselman, R.A., *et al., Mol. Cell Biol., 7*:1797-1806 (1986). One approach to doing so is to use mutated proviral genome to develop retroviral packaging cell lines. However, production of helper virus and/or transfer of packaging function (i.e., viral genes) may still occur.

Thus, an improved retroviral packaging cell line is needed which limits the potential for generation of helper virus.

Summary of the Invention

The present invention relates to a stable packaging cell line which produces helper-free pseudotyped retroviruses and is of mammalian origin, preferably of non-murine origin, such as stable packaging human cell lines. These are referred to herein respectively, as stable pseudotyped retrovirus packaging cell lines and stable pseudotyped retrovirus packaging human cell lines. The packaging cell line comprises one or more non-retroviral expression constructs, such as an expression construct with the human cytomegalovirus (CMV) immediate early promoter or derivatives of this promoter (e.g., pMD), which direct expression of: a) the retroviral gag gene and the retroviral pol gene, referred to as the retroviral *gagpol* genes, and b) a non-retroviral gene which is under the control of an inducible operator system and whose gene

product pseudotypes retroviral core virions. The *gagpol* gene products package the desired recombinant RNA into core virions, which are pseudotyped by the non-retroviral gene product, resulting in production of a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant retrovirus.

In one embodiment, the present invention relates to a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range. These cell lines generate helper-free recombinant pseudotyped retrovirus. The packaging cells comprise one or more non-retroviral expression constructs which direct expression of retroviral *gagpol* genes and a gene for the Vesicular Stomatitis Virus G (VSV-G) glycoprotein. The VSV-G glycoprotein, which is under the control of an inducible operator system (e.g., *tet* operator), provides an envelope protein that pseudotypes the retroviral core virion generated by the gagpol proteins. The result is a stable, pseudotyped retrovirus packaging cell line (e.g., H29 gagpol) which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range. In another embodiment, altered (e.g., mutated) retroviral *gagpol* genes are used to produce a stable, pseudotyped retrovirus packaging cell line (e.g., H29 new gagpol cell line).

The present invention further relates to a method of making a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus. In the method of the present invention, mammalian cells are co-transfected with one or more non-retroviral expression constructs which direct the expression of: a) retroviral *gagpol* genes and b) a non-retroviral gene which is under the control of an inducible operator system and provides a pseudotyped envelope for retroviral core virions. The gagpol proteins package the

desired recombinant RNA into core virions, which are pseudotyped by the non-retroviral gene product, resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus.

In one embodiment, the present invention relates to a method of making a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range. In this embodiment, mammalian cells are co-transfected with one or more non-retroviral expression constructs which direct the expression of retroviral *gagpol* genes (e.g., wild type or altered) and a VSV-G gene. The VSV-G gene, which is under the control of an inducible operator system, provides a pseudotyped envelope protein for the retroviral core virions, which are produced by the gagpol proteins. This results in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range.

In another embodiment, the present invention relates to a method of making a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range. In the method, mammalian host cells are co-transfected with a first non-retroviral construct which expresses the gene for *tet* transactivator fusion protein (tTA) (Gossen, M. and Bujard, M., *Proc. Natl. Acad. Sci.*, *89*:5547-5551 (1992)) and a second non-retroviral construct which expresses a gene for the VSV-G glycoprotein under the control of *tet* operator (minimal human CMV immediate early promoter incorporating *tet* binding sequences). The transfected cells are screened for tetracycline-inducible VSV-G expression; VSV-G protein is not detected in the presence of tetracycline and is detected in the absence of

tetracycline. Such cells are transfected with a third non-retroviral construct which expresses the retroviral *gagpol* genes, and screened for production of retroviruses. Transfected cells which produce retroviruses are stable, pseudotyped retrovirus packaging cells which generate helper-free recombinant pseudotyped retrovirus with a pantropic host range.

The present invention further relates to the particular packaging cell lines described herein (H29 gagpol or 293GPG cell line, H29 new gagpol cell line) and the particular cells and constructs (e.g., packaging elements) used to produce the stable, pseudotyped retrovirus packaging cell line described herein (H29 cells and pMD, pMDtet, pMDtet.G, PMD.gagpol, pMD.new gagpol constructs).

Another aspect of the invention is a retroviral vector for producing a cDNA library for expression in mammalian cells. The retroviral vector comprises two retroviral long terminal repeats (LTRs) (e.g., a 5′ retroviral LTR and a 3′ retroviral LTR), a cloning site for insertion of cDNA and a cytomegalovirus (e.g., human) promoter. In one embodiment, the two LTRs are Moloney murine leukemia virus (MMLV). In a specific embodiment, the 3′ MMLV LTR is unmodified and the 5′ is a modified or chimeric MMLV LTR in which the U3 region of the 5′ MMLV LTR is replaced with the cytomegalovirus (e.g., human) promoter or the cytomegalovirus enhancer-promoter.

The present invention also relates to a cDNA library for expression in mammalian cells. The library comprises retroviral vectors which comprise two retroviral LTRs, cDNA and a cytomegalovirus promoter. The cDNA is positioned at a unique cloning site within the retroviral vector, preferably between the two LTRs, and is operably linked to the cytomegalovirus promoter.

The present invention also relates to a method of expression cloning in mammalian cells. The method comprises the steps of introducing into mammalian cells a cDNA expression library comprising retroviral vectors of the present invention and maintaining the mammalian cells containing the expression library under conditions appropriate for expression of the cDNA expression library, whereby the cDNAs in the expression library are expressed in the mammalian cells. In one embodiment, the cDNA expression library is introduced into mammalian cells by infection with pseudotyped retroviruses produced in a stable mammalian (e.g., human, murine) packaging cell line. The stable mammalian packaging cell line can be selected to produce pseudotyped retroviruses with pantropic, ecotropic or amphotropic host range, preferably pantropic host range. In a particular embodiment, the present invention relates to a method of expression cloning in mammalian cells. The method comprises the steps of introducing a cDNA expression library comprising retroviral vectors which comprise two retroviral LTRs, cDNA and a cytomegalovirus promoter into a packaging cell line which produces pseudotyped retroviruses; maintaining the packaging cell line containing the expression library under conditions appropriate for generation of pseudotyped retroviral particles containing the cDNA expression library; infecting mammalian cells with the pseudotyped retroviral particles, under conditions appropriate for infection of the mammalian cells; and maintaining the resulting mammalian cells under conditions appropriate for expression of the cDNA in the mammalian cells. In a particular embodiment, the packaging cell line is a stable human embryonic kidney cell line and, specifically, a human 293-derived cell line.

The present invention also relates to a method of cDNA expression cloning in mammalian cells, wherein VSV-G pseudotyped retrovirus particles which contain RNA produced

by transcription of cDNA in a cDNA library are produced in a packaging cell line. The cDNA library comprises vectors which, in turn, comprise two retroviral LTRs, cDNA and a cytomegalovirus promoter; the cDNA is positioned between the LTRs and operably linked to the promoter. Mammalian cells are then infected with the VSV-G pseudotyped retroviral particles produced under conditions appropriate for transcription of RNA contained in the VSV-G pseudotyped retrovirus particles and production of protein encoded by cDNA in the cDNA library (by translation of the RNA in the retrovirus particles) in the mammalian cells. Mammalian cells which contain the RNA contained in the VSV-G pseudotyped retrovirus particles or protein encoded by the cDNA in the cDNA library are detected, using known methods. For example, RNA can be detected using *in situ* hybridization. Alternatively, immunodetection can be used, where the cDNA encodes protein which is expressed at the cell surface and the expressed proteins can be detected using antibodies which bind the protein expressed by the cDNA of interest (see e.g., U.S. Patent No. 5,506,126). In addition, epitope tags can be used to detect the protein expressed. In addition, functional assays can be used to detect the function of a protein expressed by the cDNA of interest (e.g., a protein which confers an adhesive phenotype on a cell).

The present invention also relates to a method of identifying a gene defect responsible for a mutant phenotype using cDNA expression cloning by complementation in mammalian cells. In this method, VSV-G pseudotyped retrovirus particles which contain RNA produced by transcription of cDNA in a cDNA library are produced in a packaging cell line. The cDNA library comprises two retrovirus LTRs, cDNA and a cytomegalovirus promoter; the cDNA is positioned between the LTRs and operably linked to the promoter. Mammalian cells which display a mutant

phenotype are infected with the VSV-G pseudotyped retroviral particles under conditions appropriate for transcription of RNA contained in the VSV-G pseudotyped retrovirus particles and production of protein encoded by cDNA in the cDNA library (by translation of the RNA in the retrovirus particles). Mammalian cells with the mutant phenotype which display the wild type phenotype upon expression of the cDNA are identified. The cDNA which confers the wild type phenotype in the mammalian cells is then identified, thereby determining the gene defect responsible for the mutant phenotype.

Development of the stable, pseudotyped retrovirus packaging cell lines described herein limits the formation of helper virus. As a result, stable pseudotyped retrovirus packaging cell lines are particularly valuable reagents for *in vivo* gene transfer studies aimed at cell lineage analysis and the development of human gene replacement therapies.

Use of the retroviral vectors of the present invention enables retroviral cDNA expression cloning in any mammalian cell type, obviating the need for specialized cells for efficient expression cloning. In any mutant mammalian cell type for which there is a phenotype distinct from the wild-type parental cell type (e.g., primary human cells derived from patients, primary or established cell lines derived from mutant animal strains, primary or established cell lines derived from knockout mice, mutant cell lines generated in cell culture) the genetic difference between the mutant and wild type cell (the genetic alteration(s) or defect(s)) can be rapidly identified by expression cloning by complementation using this invention.

Brief Description of the Figures

Figure 1 is a schematic representation of the pMD construct.

Figure 2 is a schematic representation of the pMDtet construct.

Figure 3 is a schematic representation of the pMD.G construct.

Figure 4 is a schematic representation of the pMDtet.G construct.

Figure 5 is a schematic representation of the pMD.gagpol construct.

Figure 6 is a schematic representation of the pMD.new gagpol construct.

Figure 7 is a schematic representation of the plasmid, pBC.tTA

Figure 8 is a schematic representation of the plasmid, MFG.SnlsLacZ.

Figure 9A is a schematic representation of the ΔU3 retroviral construct, ΔU3nlsLacZ

Figure 9B is a schematic representation of the ΔU3 retroviral construct, ΔU3Bam.

Detailed Description of the Invention

The present invention relates to a stable retrovirus packaging cell line of mammalian origin, preferably of non-murine origin, such as stable packaging human cell lines, which produce pseudotyped retroviruses. These are referred to herein, respectively, as stable pseudotyped retrovirus packaging cell lines and stable pseudotyped retrovirus packaging human cell lines for producing pseudotyped retroviruses for retroviral gene transfer. The packaging cell lines of the present invention comprise one or more non-retroviral constructs for expression of retroviral gagpol proteins, which produce a retroviral core virion, and a protein which provides a pseudotyped envelope for the

retroviral core virion. The protein which provides a pseudotyped envelope for retroviral core proteins is under control of an inducible operator system. That is, production of retrovirus by the packaging cell line described herein is controlled by the inducible expression of the protein which provides a pseudotyped envelope for a retroviral core virion. Packaging cell lines of the present invention remain viable when uninduced (e.g., in the presence of tetracycline when a *tet* operator is used) and express retroviral gagpol proteins which are non-enveloped; the uninduced packaging cell lines are capable of generating (producing) recombinant pseudotyped retroviral particles when induced (e.g., in the absence of tetracycline when a *tet* operator is used). Thus, the pseudotyped retrovirus packaging cell line of the present invention is stable. Once induced, the packaging cell lines generate (produce) recombinant pseudotyped retroviral particles.

In addition, the packaging cell line of the present invention limits the potential for generation of helper virus. Use of non-retroviral constructs and a non-retroviral protein which produces a pseudotyped envelope for the retroviral core virion contributes to the limited generation of helper virus. Potential for helper virus formation can be further limited by using non-murine cells (e.g., human cells). Murine cell lines (e.g., NIH 3T3 cells) are typically used to generate retrovirus packaging cell lines. However, the presence of endogenous murine retrovirus in the genome of murine cell lines, such as NIH 3T3 cells (Danos, *et al.*, *Proc. Natl. Acad. Sci.*, 85:6460-6466 (1988), could facilitate recombination events between the host cell genome, the retroviral expression constructs and the retroviral vectors, thereby contributing to production of helper virus.

In addition, the retroviral *gagpol* genes can be altered (e.g.,mutated), further limiting the potential for the production of helper virus. An example of mutated *gagpol* sequences (i.e., new 5′ gagpol; new 3′ gagpol) is described in Example 1.

As described in Example 2, packaging cell lines of the present invention can be derived from human 293 cells which incorporate a novel non-retroviral, human CMV immediate early promoter expression construct (pMD) to express the *gagpol* gene and pseudotyped envelope which limits the potential for generation of helper virus. In addition, silent mutagenesis of *gagpol* coding sequences minimizes homology with retroviral vector sequences, further limiting the potential for generation of helper virus. As further described in herein, the packaging cell lines of the present invention express the Vesicular Stomatitis Virus G (VSV-G) glycoprotein which efficiently pseudotypes the retroviral core virions. The VSV-G glycoprotein has a broad host range. Therefore, VSV-G pseudotyped retroviruses demonstrate a broad host range (pantropic) and are able to efficiently infect cells that are resistant to infection by ecotropic and amphotropic retroviruses (Yee, J.-K., *et al.*, *Proc. Natl. Acad. Sci.*, *91*:9564-9568 (1994)). High levels of expression of VSV-G are cytotoxic and therefore, VSV-G expression in the new packaging cell line is controlled by an inducible operator system, such as the inducible *tet* operator system, allowing for tight regulation of gene expression (i.e., generation of retroviral particles) by the concentration of tetracycline in the culture medium.

Finally, as demonstrated herein, VSV-G pseudotyped retroviral particles can be concentrated more than 100-fold by ultracentrifugation (Burns, J.C., *et al.*, *Proc. Natl. Acad. Sci.*, *90*:8033-8037 (1993)). The stable VSV-G pseudotyped retrovirus packaging cell lines permit

generation of large scale viral preparations (e.g. from 10 to 50 liters supernatant) to yield retroviral stocks in the range of 10^7 to 10^{11} retroviral particles per ml.

The H29 cells that express the inducible VSV-G protein have been observed in cell culture for better than 20 passages. The H29 cells at passage 20 remain viable and continue to express in an inducible manner detectable VSV-G protein (e.g., by cell fusion studies, Western blotting) at levels equivalent to cells at an early passage.

The expression construct for use in the present invention is a non-retroviral vector which directs expression of retroviral *gagpol* genes used to produce a retroviral core virion, and a protein which provides a pseudotyped envelope for the retroviral core virion. As described in Example 1, a suitable expression construct for use in the present invention is a human cytomegalovirus (CMV) immediate early promoter construct. Other examples of constructs which can be used to practice the invention include constructs that use SV40, RSV and rat β-actin promoters.

One or more of the non-retroviral expression constructs can be used to express the *gagpol* genes and the protein which provides a pseudotyped envelope, using skills known in the art. For example, the proteins can be expressed using one non-retroviral expression construct. In addition, two non-retroviral expression constructs can be used wherein one construct expresses the *gagpol* genes and the other construct expresses the genes (VSV-G, tTA) which provide a pseudotyped envelope under control of an inducible operator. Alternatively, as described in Example 1, three non-retroviral constructs can be used: the first non-retroviral construct codes for the inducible *tet* transactivator protein (tTA) which controls expression of the gene that expresses a pseudotyped envelope, the second

non-retroviral construct expresses the genes which provides the pseudotyped envelope and the third non-retroviral construct expresses the *gagpol* genes. Further, the *gag* and *pol* sequences can be expressed separately, requiring a fourth non-retroviral construct (e.g., in which the third retroviral construct expresses the *gag* gene and the fourth retroviral expression construct expresses the *pol* gene).

As referred to herein, a "pseudotype envelope" is an envelope protein other than the one that naturally occurs with the retroviral core virion, which encapsidates the retroviral core virion (resulting in a phenotypically mixed virus). A suitable protein which provides a pseudotyped envelope is the Vesicular Stomatitis Virus G (VSV-G) glycoprotein, as described in Example 1. Any suitable serotype (e.g., Indiana, New Jersey, Chandipura, Piry) and strain (e.g., VSV Indiana, San Juan) of VSV-G can be used in the present invention. The protein chosen to pseudotype the core virion determines the host range of the packaging cell line. VSV-G interacts with a specific phospholipid on the surface of mammalian cells (Schlegel, R., *et al.*, *Cell*, *32*:639-646 (1983); Supertzi, F., *et al.*, *J. Gen Virol.*, *68*:387-399 (1987)). Thus, the packaging cell line which utilizes VSV-G to provide a pseudotyped envelope for the retroviral core virion has a broad host range (pantropic). Other suitable proteins which can be used to provide a pseudotyped envelope for a retroviral core virion include type C murine retroviral envelope proteins; HTLV-1 envelope protein, Gibbon ape leukemia virus envelope protein, and derivatives of a suitable protein which provide a pseudotyped envelope (e.g., proteins which include insertions, deletions or mutations to prepare targeted envelope sequences such as ecotropic envelope with the EPO ligand, synthetic and/or other hybrid envelopes; derivatives of the VSV-G glycoprotein). In addition, derivatives of murine retroviral envelope proteins can be

used. For example, derivatives of the VSV-G protein can be obtained in which the portion of the VSV-G protein which is responsible for binding to the cell surface is replaced by a specific ligand and the portion of the VSV-G protein responsible for membrane fusion is retained. The portion of the VSV-G protein responsible for binding to the cell surface is determined for example, by performing point mutation and deletion sequence analysis of the VSV-G sequence. The ability of each mutated VSV-G protein to bind to the cell surface is determined using an appropriate binding assay. Retroviral particles incorporating such derivatives of VSV-G protein would now be able to be targeted to specific cell populations.

As discussed above, an inducible operator is used for controlled expression of the gene which provides a pseudotyped envelope. For example, high levels of VSV-G expression are cytotoxic (Yee, J.-K., *et al.*, *Proc. Natl. Acad. Sci.*, *91*:9564-9568 (1994)). Thus, an inducible tetracycline (i.e., *tet*) operator system is used to allow for tight regulation of VSV-G expression by the concentration of tetracycline in the culture medium of the packaging cell line. That is, with the *tet* operator system, in the presence of tetracycline, the tetracycline is bound to the *tet* transactivator fusion protein (tTA), preventing binding of tTA to the *tet* operator sequences and allowing expression of the gene under control of the *tet* operator sequences (Gossen, M. and Bujard, M., *Proc. Natl. Acad. Sci.*, *89*:5547-5551 (1992)). In the absence of tetracycline, the tTA binds to the *tet* operator sequences preventing expression of the gene under control of the *tet* operator. Examples of other inducible operator systems which can be used for controlled expression of the protein which provides a pseudotyped envelope are 1) inducible eukaryotic promoters responsive to metal ions (e.g., the

metallothionein promoter), glucocorticoid hormones and 2) the *lac* repressor/operator/inducer system of *E. coli*.

The nucleotide sequences which are encoded by the non-retroviral constructs can be obtained from a variety of suitable sources for use in the present invention. For example, nucleotide sequences expressing the operator system, the pseudotyped envelope and the *gagpol* sequences can be purified from natural sources, produced by chemical synthesis or produced by recombinant DNA techniques. For example, as described in Example 1, the *gagpol* sequence can be obtained using the pCripenv construct.

The cells used to prepare the packaging cells are mammalian cells, preferably non-murine cells. In a particular embodiment, the cells used to produce the packaging cell line are human cells (e.g., 293 cells, Graham, F., *et al.*, *J. Gen. Virol.*, *36*:59-72 (1977); tsa 201 cells, Heinzel, S., *et al.*, *J. Virol.*, *62*:3738 (1988)).

The packaging cell lines of the present invention can be used to produce recombinant pseudotyped retroviruses to enable gene transfer, *in vitro* and *in vivo*, for purposes of expressing all or a portion of a desired gene in eukaryotic cells. For example, using known techniques, the packaging cell lines described herein can be used to produce recombinant pseudotyped retroviruses which are used to introduce a gene which encodes a particular mRNA, protein or polypeptide (e.g., therapeutic proteins or polypeptides, such as insulin, human growth hormone, erythropoietin, gene replacement for cystic fibrosis (CFTR), familial hypercholesterolemia (LDL receptor), ADA Deficiency (ADA), Gaucher's Disease (glucocerebrosidase), antisense therapy by expression of inhibitory mRNA sequences) into eukaryotic cells in order to produce the mRNA or protein in quantities which are useful in administration for therapeutic purposes or in a diagnostic context (Yee, J.-K., *et al.*, *Proc. Natl.*

Acad. Sci., *91*:9564-9568 (1994); Dranoff, G., *et al.*, *Proc. Natl. Acad. Sci.*, *90*:3539-3543 (1993); Miller, A.D., *et al.*, *Meth. in Enz.*, *217*:581-599 (1993)). That is, pseudotyped recombinant virus can be harvested from the packaging cell lines and used as viral stock to infect recipient cells in culture or *in vivo* using known methods. In the case of secreted proteins or proteins expressed in hematopoietic cells, sensitive assays such as ELISA or Western blotting can be used to assess gene transfer efficiency. Alternatively, high titer viral stocks produced by packaging cell lines provide superior gene transfer efficiency in transducing cells (e.g., hematopoietic cells) and reduce contamination as compared with current co-cultivation techniques.

The packaging cell lines of the present invention can also be used to produce pseudotyped retroviruses containing DNA of interest for introducing DNA or genes of interest into mammalian cells, such as human cells, which will subsequently be administered into localized areas of the body (e.g., *ex vivo* infection of autologous white blood cells for delivery of protein into localized ares the body, see e.g., U.S. Patent No. 5,399,346).

In addition, the packaging elements used to generate the stable, pseudotyped retrovirus packaging cell can be used in a variety of ways. For example, the H29 cell line, which demonstrates inducible VSV-G expression, can be used to generate retroviral libraries for expression cloning. The potential for production of high titer viral stocks will improve the representation of rare cDNAs in a given library. The packaging cell lines of the present invention can also serve as the basis for further generation of pseudotyped packaging cell lines.

The packaging elements can be used as expression constructs for purposes of efficient constitutive (e.g., pMD) and inducible (pMDtet) gene expression. The pMD*tet*.G

construct can be used for other applications for inducible expression of VSV-G. The pMD gagpol and pMD new gagpol can be used to develop new generations of retrovirus packaging cell lines. The pMD, pMDtet, pMDtet.G and pMD.gagpol constructs can be used for efficient expression of a heterologous gene.

Thus, as described herein, a stable cell line expressing VSV-G in an inducible fashion has been generated. In addition, a new CMV expression vector (pMD) and its derivatives (pMD.G, pMDtet.G, pMD.gagpol, pMD.new gagpol) which use genomic human β-globin sequences for high levels of expression has been developed. As further described herein, a stable 293-based packaging cell line that uses CMV expression constructs, as compared with mutated proviral constructs, which limit the potential for helper virus has been developed and use of a mutated gagpol expression construct in a stable cell line to limit the potential for helper virus has been demonstrated.

The present invention also relates to a retroviral vector for producing a cDNA expression library, for expression in mammalian cells, comprising two retroviral LTRs, a cloning site for insertion of cDNA, and a cytomegalovirus promoter. In one embodiment, the two LTRs are Moloney murine leukemia virus (MMLV). In a specific embodiment, the 3′ MMLV LTR is unmodified and the 5′ is a modified or chimeric MMLV LTR in which the U3 region of the 5′ MMLV LTR is replaced with the cytomegalovirus promoter or the cytomegalovirus enhancer-promoter.

The retroviral LTRs can be derived from any suitable retroviral vector, preferably a retroviral vector which results in high titer or expression of retroviral proteins The two LTRs can be derived from the same retroviral vector or different retroviral vectors. For example, as described in Example 3, both retroviral LTRs can be derived from the Moloney murine leukemia virus (MMLV). Other suitable

retroviral LTRs include, for example, those derived from murine sarcoma virus (MSV), murine papillary sarcoma virus (MPSV), and Friend virus.

The cloning site of the retroviral vector can be a variety of cloning sites. For example, as described in Example 3, the cloning site can be a BamHI cloning site. Other suitable cloning sites for use in the retroviral vectors of the present invention include, for example, any unique or infrequent restriction site within the gagpol or env genome, or within the U3 region.

The cytomegalovirus promoter can be obtained from any suitable source. For example, as described in Example 3, the complete cytomegalovirus enhancer-promoter is derived from the human cytomegalovirus (HCMV). Part or all of previously described CMV promoter could be used in the present invention. Other suitable sources for obtaining a cytomegalovirus promoter include commercial sources, such as Clontech, Invitrogen and Stratagene.

The retroviral vectors of the present invention can be used for expression cloning in mammalian cells, wherein a cDNA expression library comprising the retroviral vectors described herein are introduced into mammalian cells under conditions appropriate for expression of the cDNA expression library. In one embodiment, the present invention relates to a cDNA expression library for expression in mammalian cells, wherein the library comprises two retroviral LTRs, a cytomegalovirus promoter and cDNA, wherein the cDNA is positioned between the retroviral LTRs and is operably linked to the cytomegalovirus promoter.

The cDNA for use in the present invention is any cDNA which is of interest for expression in mammalian cells. The cDNA can be from any type of cells, such as blood cells, cells from tissue samples, or cultured cells. Generally the cDNA will be from the same type of cell in

which the cDNA is being expressed in those cases where expression cloning by complementation is being carried out.

The cDNA library for use in the present invention can be obtained using routine methods (e.g., Seed and Aruffo, *Proc. Natl. Acad. Sci, USA, 84*:3365-3369 (1987)). For example, mRNA can be prepared from any cell and the cDNA synthesized using standard techniques (Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989)) and commercially available cloning kits (e.g., Pharmacia, Invitrogen, Stratagene).

The retroviral vectors of the present invention can be introduced into mammalian cell using any technique which results in expression of the cDNA expression library in the cell (e.g., electroporation, calcium phosphate precipitation, cationic lipids, liposomes). In one embodiment, the cDNA expression library is introduced into a packaging cell line to produce retroviral particles, containing RNA transcribed from the cDNA expression library, which are used to infect mammalian cells resulting in expression of the cDNA expression library in the infected mammalian cell. In this embodiment, a packaging cell line (e.g., the 293GPG packaging cell line described herein) can be used to produce pseudotyped retroviral particles useful for expression cloning in mammalian cells, as described in Example 4. Other suitable packaging cell lines for use in the present invention include other human cell line derived (e.g., embryonic cell line derived) packaging cell lines and murine cell line derived packaging cell lines, such as Psi-2 cells (Mann, R., *et al., Cell, 33*:153-159 (1983); FLY (Cossett, F.L., *et al., Virol., 193*:385-395 (1993)), BOSC 23 cells (Pear, W.S., *et al., Proc. Natl. Acad. Sci, USA, 90*:8392-8396 (1993), PA317 cells (Miller, A.D. and C. Buttimore, *Molec. and Cell. Biol., 6*:2895-2902 (1986)), Kat cell line, (Finer, M.H., *et al., Blood, 83*:43-50 (1994)) GP+E-86 cells and GP+EM12

cells (Markowitz, D., *et al.*, *J. Virol.*, *62*:1120-1124 (1988), and Psi Crip and Psi Cre cells (U.S. Patent No. 5,449,614; Danos, O. and Mulligan, R.C., *PNAS, USA, 85*:6460-6464 (1988)). Also see Yang, Y., *et al.*, *Human Gene Ther.*, *6*:1203-1213 (1995). The packaging cell lines for use in the present invention can produce retroviral particles having a pantropic amphotropic or ecotropic host range. Therefore, in this embodiment, the cDNA expression library of the present invention can be expressed in any cell within the host range of the retroviral particle produced by the packaging cell line. Further, in this embodiment, the promoter of the retroviral vector of the present invention can be any promoter which produces sufficient levels of transcription of the retroviral vector in the particular packaging cell line (e.g., SV40 promoter, RSV promoter, β-actin promoter).

The retroviral vectors of the present invention (e.g., the ΔU3 retroviral vectors) can be used for transient transfection of packaging cell lines which produce pseudotyped retroviruses (e.g., the 293 GPG cells described herein, which are also referred to herein as H29 gagpol cells) for production of high titer pseudotyped (e.g., VSV-G) retrovirus. The retroviral vectors of the present invention permit construction of cDNA expression libraries (in the retroviral vectors) for transfection of retroviral packaging cell lines, in which pseudotyped retrovirus particles containing the cDNA expression libraries are produced. Each pseudotyped retroviral particle generally contains multiple mRNA molecules.

As described in Example 4, high titer VSV-G pseudotyped virus using the ΔU3 retroviral vectors have been produced using the 293GPG cells which can be used for expression cloning by complementation in any cell line having a VSV-G host range. The retroviral vector, ΔU3nlsLZ, has been used to transfect 293GPG cells which

produce retroviruses at titers up to 3 x 10^6 infectious units (i.u.)/ml. In addition, as described in Example 5, the retroviral cDNA cloning vector, ΔU3BAM, has been used to transfect 293GPG cells with levels of expression and viral titers comparable to the ΔU3nlsLZ vector. Assessment of the effect of 5′ untranslated sequences on cDNA expression and viral titer showed that the viral ΔU3Bam vector can accommodate up to 165 base pairs of 5′ upstream non-coding DNA sequences with only a modest reduction in expression or viral titer (compared with a MFG-derived based retroviral vector which has been optimized for high cDNA expression and generation of high viral titers). The VSV-G retroviral pseudotypes produced by 293GPG cells described herein have broad host range and will permit infection of any mammalian type (Yee, J.-K, *et al.*, *PNAS*, *91*:9564-9568).

Thus, the present invention provides retroviral vectors which can be used with a variety of mammalian packaging cell lines to produce pseudotyped retroviral particles which can, in turn, be used to infect a variety of mammalian cells containing the expression product (protein, polypeptide) encoded by the cDNA. In a specific example, the retroviral vectors are used to transiently transfect 293GPG cells to produce high titer virus with a VSV-G host range. The ΔU3BAM retroviral vector will allow cloning of any cDNA library into the vector. Transfection of the retroviral cDNA library into 293GPG cells produces retrovirus at titers >10^6 i.u./ml that are capable of infecting any mammalian cell type. Infection of host cells with this retroviral vector results in stable integration of the proviral genome, facilitating long-term high level expression of the cDNA in the retroviral construct.

The novel methodology described herein makes use of retroviral vectors (e.g., ΔU3Bam retroviral vectors) and a

packaging cell line (e.g., the 293GPG cells), and provides for retroviral cDNA expression cloning in any mammalian cell type, obviating the need for specialized cells (e.g. Cos7 cells, oocytes) for efficient expression cloning. In any mutant mammalian cell type for which there is a phenotype distinct from the wild-type parental cell type (e.g., primary human cells derived from patients, primary or established cell lines derived from mutant animal strains, primary or established cell lines derived from knockout mice, mutant cell lines generated in cell culture) the gene defect(s) can be rapidly identified by expression cloning by complementation using this invention.

An extension of this methodology is the use of the retroviral vectors of the present invention to produce pseudotyped retrovirus in the packaging cells, for expression of candidate cDNA clones in cells that are derived from patients with genetic defects and established phenotypes. This will permit screening to determine the basis for genetic defects (e.g., altered expression of a gene involved in metabolism) in patients by complementation analysis. For example, numerous patients have been characterized biochemically and genetically to have single gene defects in fatty acid metabolism. However, the mutant genes have not been established by conventional methods. A panel of retroviral constructs which encode candidate cDNAs for various enzymes in fatty acid metabolism can be tested, for example, for complementation in primary fibroblasts from these patients.

The retroviral vectors of the present invention are retroviral-derived vectors (e.g., Moloney murine leukemia virus-derived vectors) in which the retroviral enhancer-promoter (e.g., HCMV) has been precisely replaced with the cytomegalovirus enhancer-promoter in order to facilitate high levels of expression in the packaging cell lines (e.g., 293GPG).

Further, the retroviral vectors of the present invention can be used to generate retroviral cDNA expression libraries to allow expression cloning in any mammalian cell type. In addition, identification of mutant genes responsible for human genetic defects by expression cloning by complementation can be accomplished using retroviral vectors of the present invention. Expression cloning of mutant genes from cultured cell lines that have been mutagenized in culture and have a known phenotype or from primary or established cell lines derived from animals with mutant phenotypes can be performed. For example, the expression cDNA library of the present invention is introduced into a cell having a mutated phenotype and identification of the gene(s) which complements the defect is determined. Thus, an important advantage provided by the present invention is that expression cloning by complementation as described herein can be used to identify gene(s) responsible for a phenotype caused by a mutation and obtain proof of the function of the responsible gene(s). Further, the example of the present invention will allow identification in knockout mice of gene products which complement the introduced mutation, or of gene products which function within the pathway(s) affected by the mutation (i.e., identification of downstream effectors by suppressor analysis) can be determined with the retroviral vectors of the present invention. Retroviral cDNA libraries constructed in the retroviral vectors for commercial distribution are also provided.

The invention is further illustrated by the following examples, which are not intended to be limiting in any way.

Exemplification

Example 1 Expression Vector Constructions

pMD (see Figure 1) was constructed with the 3.1 kb EcoRI-BamHI fragment from pBC12.AB that includes the pXF3 backbone and human CMV immediate early promoter regions and a 1.34 kb BamHI-Xbal fragment derived from pUCMdβs(R)S (Sadelain, M., *et al., Proc. Natl. Acad. Sci. USA, 92*:6728-6732 (1995)) that includes the genomic human β-globin sequences from the BamHI site in exon 2 through 690 bp in the 3′ untranslated region. The plasmid pUCMdβs(R)S, however, differs from the genomic sequence in that there is a 374 bp deletion in the second intron between the first and third RsaI sites. pBC12.AB is a derivative of pBC12/CMV/IL-2 (B. Cullen, *Cell 46*:973 (1986)) in which the IL-2 sequences (bp 756-1439) have been replaced with a polylinker. The 3.1 kb EcoRI-BamHI and 1.34 kb BamHI-Xbal fragments were ligated after the EcoRI and Xbal overhangs were blunt-ended by treatment with the Klenow fragments.

pMD.G (see Figure 3) was constructed with a 1.6 kb EcoRI fragment containing the VSV G gene that was derived from pSVGL (Rose and Bergman, *Cell 34*:513 (1983)) and was cloned into the EcoRI site in pMD which is within exon 3 of the genomic human β-globin sequence.

pMDtet (see Figure 2) was generated with a 0.47 kb XhoI-BamHI fragment from pUHC 13-3 (Gossen and Bujard, *Proc. Natl. Acad. Sci. 89*:5547-5551 (1992)), which contains the tet operator and minimal CMV promoter sequences, the 1.34 BamHI-Xbal fragment from pUCMdβs(R)S and a 3.06 kb Xbal-XhoI fragment from pSL301 (Invitrogen).

To construct pMDtet.G (see Figure 4), the 1.6 kb EcoRI fragment containing the VSV G gene (pSVGL) was cloned into the EcoRI site in pMD.tetG which is within exon 3 of the genomic human β-globin sequence.

To construct pMD.gagpol (see Figure 5), PCR was performed with pCRIPenv- (Danos and Mulligan, *Proc. Natl. Acad. Sci., 85*:6460-6466 (1988)) and the following pairs of primers: 5′-CGGAATTCATGGGCCAGACTGTTACC-3′ (SEQ ID No:1) and 5′-AGCAACTGGCGATAGTGG-3′ (SEQ ID No:2), 5′-CGGAATTCTTAGGGGGCCTCGCGG-3′ (SEQ ID No:3) and 5′-ACTACATGCTGAACCGGG-3′ (SEQ ID No:4). The PCR products were digested with EcoRI and XhoI and with EcoRI and HindIII, respectively, to generate 0.94 kb EcoRI-XhoI and 0.94 kb HindIII-EcoRI fragments. These fragments were ligated with the 3.3 kb XhoI-HindIII fragment from pCRIPenv- and pUC19 which had been linearized with EcoRI and phosphatase treated to produce pUC19.gagpol. The 5.2 kb EcoRI fragment from pUC19.gagpol was cloned into the EcoRI site in pMD, which is within exon 3 of the genomic human β-globin sequence, to yield pMD.gagpol.

To construct pMD.new gagpol (see Figure 6), PCR was performed with pBCIL2.gagpol (Chung and Mulligan, unpublished results), which encodes a mutated gagpol sequence and the following pairs of primers: 5′-CGGAATTCATGGGTCAGACTGTTACTAC-3′ (SEQ ID No: 5) and 5′-AGCAACTGGCGATAGTGG-3′ (SEQ ID No: 2), 5′-CGGAATTCTTAGGGAGCTTCTCTTGTTAG-3′ (SEQ ID No: 6) and 5′-ACTACATGCTGAACCGGG-3′ (SEQ ID No: 4). The mutated gagpol sequences are as follows:

New 5′gagpol:
5′-
ATGGGTCAGACTGTTACTACCCCTCTAAGTTTAACTTTGGGCCATTGGAAAGATGTAGA
GAGGATCGCCCACAACCAGAGTGTAGACGTTAAGAAAAGACGTTGGGTCACTTTTTGTT
CTGCAGAGTGGCCTACCTTCAACGTAGGCTGGCCAAGAGATGGTACTTTTAACAGAGAC
CTTATTACCCAGGTCAAGATCAAAGTTTTTAGTCCAGGCCCTCACGGACATCCAGATCA
GGTCCCTTACATTGTCACCTGGGAAGCTCTTGCCTTTGACCCTCCCCCTTGGGTGAAGC
CTTTTGTCCACCCTAAGCCCCCACCTCCCTTGCCTCCAAGTGCTCCTTCCCTCCCTCTT

GAACCCCCTCGCAGTACTCCACCTCGATCCAGTCTCTATCCTGCCCTA-3' (SEQ ID No: 7)

New 3' gagpol:

5'-

ATTCTATACGGAGCCCCTCCCCCTTTAGTTAACTTTCCAGACCCTGATATGACTAGAGT
AACAAACTCTCCTAGTCTTCAGGCACACCTCCAAGCCCTGTACCTAGTCCAACATGAAG
TGTGGAGACCCTTAGCAGCTGCATACCAGGAACAGCTTGACAGGCCTGTAGTCCCCCAC
CCGTACAGAGTGGGAGACACTGTATGGGTCCGACGCCACCAAACAAAAAACTTAGAGCC
TCGATGGAAGGGCCCCTACACTGTACTACTCACAACCCCTACAGCCCTGAAGGTTGACG
GGATAGCTGCCTGGATTCACGCTGCACACGTGAAAGCAGCTGACCCTGGAGGGGGTCCC
TCTAGCAGATTAACCTGGCGCGTACAAAGATCCCAGAATCCTCTGAAAATCAGGCTAAC
AAGAGAAGCTCCCTAA-3' (SEQ ID No: 8)

The PCR products were digested with EcoRI and XhoI and with EcoRI and HindIII, respectively, to generate 0.94 kb EcoRI-Xhol and 0.94 kb HindIII-EcoRI fragments. These fragments were ligated with the 3.3 kb Xhol-HindIII fragment from pCRIPenv- and pUC19 which had been linearized with EcoRI and phosphatase treated to produce pUC19.new gagpol. The 5.2 kb EcoRI fragment from pUC19.new gagpol was cloned into the EcoRI site in pMD, which is within exon 3 of the genomic human β-globin sequence, to yield pMD.new gagpol.

A novel CMV expression vector (pMD) was constructed for expression of the wild-type gagpol (pMD.gagpol) and for the mutagenized gagpol (pMD.new gagpol). For pMD.gagpol, reverse transcriptase assays have been performed which demonstrate production of retroviral particles under conditions of both transient and stable expression. For pMD.new gagpol, reverse transcriptase assays have been performed which demonstrate production of retroviral particles under conditions of transient expression.

Example 2 Construction of Packaging Cell Lines

H29 cell line.

Human 293 cells (gift, B. Panning; Graham, F., *et al.*, *J. Gen. Virol.*, *36*:59-72 (1977)) were grown in DMEM with 5% inactivated fetal bovine serum, supplemented with 2 mM L-glutamine, penicillin and streptomycin (293 growth media) and incubated at 37 C with 5% CO2. The 293 cells were co-transfected by the calcium phosphate precipitation method (Pear, *et al.*, *PNAS*, *90*:8392-8396 (1994)) with 5 μg pBC.tTA (see Figure 7; T. Chung and R. Mulligan, unpublished results), 5 μg pMDtet.G and 1 μg pJ6ñpuro (gift, J. Morgenstern). During transfection the 293 growth media was supplemented with 1.0 μg/ml tetracycline (Sigma). The transfected cells were plated into selection 48 hours post-transfection in 293 growth media supplemented with 1.0 μg/ml tetracycline and 2 μg/ml puromycin (Sigma). 72 independent clones were selected for clonal expansion and screened for tetracycline-inducible VSV-G expression. To screen the clones, each clone was plated in parallel into two 35 mm tissue culture dish (Corning) at 30% confluence. The following day one plate was washed twice with 2 ml 293 growth media without tetracycline and the media changed to standard 293 media supplemented with 2 μg/ml puromycin. At 48 hours the cells were harvested for total cellular protein and the paired samples run on a 7.5% SDS-polyacrylamide gel under reducing conditions. The gels were transferred onto nitrocellulose (Schleicher & Schuell, 0.45 mm) with a semi-dry electroblotter (Owl Scientific). Western blotting was performed using standard procedures. For the primary antibody a murine monoclonal anti-VSV-G IgG (Sigma) was used at a dilution of 1:800. For the secondary antibody an HRP-coupled donkey anti-mouse IgG F(ab)2 fragment (Pharmingen) was used at a dilution of 1:10,000. Chemiluminescent detection was performed with the Dupont

NEN Renaissance kit. Positive cell lines (e.g. H29) were selected on the basis of no detectable VSV-G expression in the presence of tetracycline in the growth media and the detection of inducible VSV-G expression in the absence of tetracycline in the growth media.

H29 gagpol cell line (293 GPG cell line).

H29 cells were grown in 293 growth media supplemented with 1.0 μg/ml tetracycline and 2 μg/ml puromycin (H29 media) and co-transfected by the calcium phosphate precipitation method with 10 μg pMD.gagpol linearized with ScaI and 2 μg pSV2neo. During transfection the H29 media was supplemented with 1.0 μg/ml tetracycline. The transfected H29 cells were plated into selection 48 hours post-transfection in H29 media supplemented with 1.0 μg/ml tetracycline and 0.3 mg/ml G418 (Gibco). Sixty-nine independent clones were selected for clonal expansion and were screened for reverse transcriptase activity (Goff, *et al., J. Virology, 38*:239-248 (1981)).

Of the sixty-nine independent clones selected for clonal expansion, 10 clones had reverse transcriptase activity that exceeded the positive control (i.e., Ψ Cre Cells). These 10 clones will be further characterized.

H29 new gagpol cell line

H29 cells are grown in 293 growth media supplemented with 1.0 μg/ml tetracycline and 2 μg/ml puromycin (H29 media) and co-transfected by the calcium phosphate precipitation method with 10 μg pMD.new gagpol linearized with ScaI and 2 μg pSV2neo. During transfection the H29 media was supplemented with 1.0 μg/ml tetracycline. The transfected H29 cells are plated into selection 48 hours post-transfection in H29 media supplemented with 1.0 μg/ml tetracycline and 0.3 mg/ml G418 (Gibco). Independent clones are selected for clonal expansion and screened for

reverse transcriptase activity (Goff, *et al.*, *J. Virology* (1981)).

Discussion

Thus, the derivative of pMD, pMDtet.G, provides for tetracycline inducible expression of VSV.G. Inducible expression of VSV-G has been demonstrated in a transient assay and in a stable cell line, H29. H29 is derived from 293 cells (Graham, F., *et al.*, *J. Gen. Virol.*, *36*:59-72 (1977)) and was selected after co-transfection with pBC.tTA (the Tet transactivator), pMDtet.G and pJGΩpuro. The H29 cells show inducible VSV-G expression by western blotting that is only 5-fold less than transient VSV-G expression in parental 293 cells. The H29 cells passaged in culture for 20 passages continue to demonstrate inducible VSV-G expression.

Example 3 Construction of the ΔU3 retroviral cloning vectors

The ΔU3nlsLacZ retroviral vector was constructed by precise replacement of the U3 region in the 5′ LTR of MFG.SnlsLacZ (Berns, *et al.*, *Human Gene Therapy*, *6*:347-368 (1995); see Figure 8) with the HCMV enhancer-promoter (nt -671 to -2) (Boshart, M., *Cell*, *41*:521-530 (1985)). In ΔU3nlsLacZ the entire 5′ genomic flanking region and all but 65 bp from the 3′ genomic flanking region from MFG.SnlsLacZ is eliminated.

The pMD plasmid was constructed as described in Example 1. For the construction of the ΔU3nlsLacZ, a 701 bp fragment encoding the HCMV promoter was generated by PCR with the pMD plasmid as the template with the pair of primers, 5′-GGGCCCAAGCTTCCCATTGCATACGTTGTATC-3′ (SEQ ID NO: 9) and 5′-GGACTGGCGCCGGTTCACTAAACGAGCTC-3′ (SEQ ID NO: 10), creating a 5′ *Hind* III site and a 3′ *Kas* I site. The PCR product was digested with *Hind* III and *Kas* I to

yield a 677 bp fragment. The 91 bp *Kas* I-*Sty* I was isolated from the 3' LTR of MFG (Riviere, I., *et al.*, *PNAS*, *92*:6733-6737 (1995)). The 253 bp *Sty* I-*Eag* I and the 4994 bp *Eag* I-*Sca* I fragments were isolated from MFG.SNlsLacZ (Berns, *et al.*, *Human Gene Therapy*, *6*:342-368 (1995)), and the backbone for ΔU3nlsLacZ is a 2.65 kb *Hind* III-*Sma* I fragment from pUC18.

For the construction of ΔU3Bam, a 561 bp fragment was generated by PCR with ΔU3nlsLacZ as the template with the pair of primers, 5'-GTGACCTGGGAAGCCTTGGC-3' (SEQ ID NO: 11) and 5'CGGGATCCAGTCTAGAGGATGGTCCACC-3' SEQ ID NO: 12), creating a 5' *Kas* I site and a 3' *Bam H* I site. The PCR product was digested with *Kas* I and *Bam H* I to yield a 389 bp fragment. The 389 bp *Kas* I-*Bam H* I fragment was ligated with 4466 bp *Bam H* I-*Eag* I and 695 bp *Eag* I-*Kas* I fragments that were derived from ΔU3nlsLacZ.

Figure 9A displays the structure of the ΔU3nlsLacZ vector and Figure 9B displays the structure of the ΔU3Bam vector.

Example 4 Production of VSV-G pseudotyped retrovirus by transient transfection of 293GPG cells

The plasmid pBC.tTA was constructed from pBC12/CMV/IL-2 (Cullen, B.R., *Cell*, *46*:973-982 (1986)) by replacement of the IL-2 sequences (bp 756-1439) with the tet transactivator gene from pUHD10-1 (Gossen, M., *et al.*, *Proc. Natl. Acad. Sci.*, *89*:5547-551 (1992)). Figure 7 displays the structure of the pBC.tTA plasmid.

The pMD, pMD.G, pMDtet, pMDtet.G, and pMD.gagpol constructs and the 293GPG cell line were constructed as described in Example 1.

Transient transfections with 293GPG cells were performed on 60 mm dishes where 4-5 x 10^6 cells were plated the night prior in 4 ml 293 GPG media. 4 ug of ΔU3nlsLacZ

was diluted into 300 ul Opti-MEM (Gibco BRL) and incubated at room temperature for 30 minutes with 25 ul Lipofectamine (Gibco BRL) diluted into 300 ul Opti-MEM. 2.4 ml Opti-MEM was added to the DNA-Lipofectamine mixture and layered on top of the 293GPG cells, which had been rinsed 30 minutes prior to transfection and had media replaced with 2 ml Opti-MEM. 2 ml 293 media was added at 7-8 hours post-transfection and the media was changed at 24 hours. The supernatant was harvested at 72 hours and viral titers determined as described below.

Assay for β-Galactosidase Activity and Determination of Viral Titers

To stain cells for β-galactosidase activity, cells were washed with phosphate buffered saline supplemented with 1 mM magnesium (PBS+) and fixed with 1% glutaraldehyde in PBS+ for 10 minutes at 37°C (Lim, K., *et al.*, *Biotechniques,* 7:576-579 (1989). The fixative was aspirated and the cells incubated with 3.3 mM potassium ferricyanide (Sigma), 3.3 mM potassium ferrocyanide (Sigma) and 0.2% X-gal (Molecular Probes) in PBS+ for 2 hours at 37°C. Quantitative β-galatosdase activity was determined using a commercially available luminescent assay (Clontech). To determine viral titers, NIH 3T3 cells were plated at 1 x 10^5 cells per well in 6-well culture dishes 16 hours prior to infection and incubated for 24 hours with serial dilutions of viral supernatants containing 8 ug/ml polybrene (Sigma). Viral titer was determined as the average number of cells with blue nuclei (β-galactosidase-producing cells) per twenty 1 mm^2 fields (2-3 x 10^4 cells) multiplied by a factor to account for plate size, dilution of viral stock and division of target cells in tissue culture wells.

Production of VSV-G pseudotyped retrovirus by transient transfections of 293GPG cells

The 293GPG clone was used to produce VSV-G pseudotyped retrovirus by transient transfection, taking advantage of the high transfectability of 293 cells. For transient transfection of the 293GPG cells, the ΔU3nlsLacZ retroviral construct was generated by substitution of the U3 region of the 5′ LTR of MFG.nlsLacZ with the HCMV enhancer-promoter and deletion of the genomic 5′ and 3′ flanking regions of MFG.SnlsLacZ. When compared to MFG.SnlsLacZ, ΔU3nlsLacZ enabled a 20-fold increase in expression in transient transfections. The 293GPG clone was transiently transfected by lipofectamine with an average efficiency of 40% with the ΔU3nlsLacZ construct. A 48 hour virus supernatant was collected between 24 and 72 hours post-transfection and removal of tetracycline from the growth medium. Viral titers in the range of 1-3 x 10^6 i.u./ml were achieved transiently.

Example 5 Effect of 5′ Untranslated Sequences on cDNA expression and viral titer

There are several differences between previously used vectors and the ΔU3 retroviral cloning vectors. First, the ΔU3 vectors are specifically modified for high transient expression in 293-derived cell lines (e.g. 293GPG cells) by precise replacement of the U3 region in the 5′ LTR by the complete human CMV enhancer-promoter (Boshart *et al.*, *Cell*, *41*:521-530 (1985)). Second, the ΔU3 vectors are derived from MFG which is an established high titer and high expression vector. Finally, unlike the vectors used for previous retroviral expression cloning, the effect of 5′ untranslated sequences on cDNA expression and cDNA viral titer was examined. This is important because in the course of construction of cDNA libraries (Seed, B. and

Aruffo, S., *PNAS*, *84*:3365-3369 (1987)) the cDNA inserts often include up to 200 base pairs of 5′ untranslated sequences. Therefore, the retroviral cloning vector must be able to accommodate these additional sequences so that bias will not be introduced into the library.

The effect of 5′ untranslated sequences on cDNA expression and viral titer was examined by insertion of the lacZ gene with 0-165 base pairs of 5′ untranslated sequences into the ΔU3Bam vector. The following is the data normalized to 0 base pairs of untranslated sequence (i.e., ΔU3LacZ which does not contain the modified Bam H I cloning site):

# of 5′ untranslated bp	expression (%)	viral titer (%)
0	100	100
43	51	34
106	36	31
165	19	27

These results demonstrate that the ΔU3Bam vector can accommodate up to 165 bp of 5′ untranslated sequences within the cDNA insert with only a modest reduction in expression or viral titer as compared with a non-cloning MFG-based retroviral vector. This data supports the capability of the ΔU3Bam vector to promote efficient packaging of the cDNA inserts and efficient transfer of the cDNA to the target calls.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are

intended to be encompassed in the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH
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(ii) TITLE OF INVENTION: STABLE PACKAGING CELL LINE PRODUCING PSEUDOTYPED RETROVIRUSES

(iii) NUMBER OF SEQUENCES: 12

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(A) APPLICATION NUMBER:
(B) FILING DATE:
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(C) CLASSIFICATION:

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(B) FILING DATE: November 8, 1995
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGAATTCAT GGGCCAGACT GTTACC 26

(2) INFORMATION FOR SEQ ID NO:2:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGAATTCTT AGGGGGCCTC GCGG 24

(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTACATGCT GAACCGGG 18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGGAATTCAT GGGTCAGACT GTTACTAC 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGGAATTCTT AGGGAGCTTC TCTTGTTAG 29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 402 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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ATTACCCAGG TCAAGATCAA AGTTTTTAGT CCAGGCCCTC ACGGACATCC AGATCAGGTC 240

CCTTACATTG TCACCTGGGA AGCTCTTGCC TTTGACCCTC CCCCTTGGGT GAAGCCTTTT 300

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CCTCGCAGTA CTCCACCTCG ATCCAGTCTC TATCCTGCCC TA 402

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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TGGAGACCCT TAGCAGCTGC ATACCAGGAA CAGCTTGACA GGCCTGTAGT CCCCCACCCG 180

TACAGAGTGG GAGACACTGT ATGGGTCCGA CGCCACCAAA CAAAAAACTT AGAGCCTCGA 240

TGGAAGGGCC CCTACACTGT ACTACTCACA ACCCCTACAG CCCTGAAGGT TGACGGGATA 300

GCTGCCTGGA TTCACGCTGC ACACGTGAAA GCAGCTGACC CTGGAGGGGG TCCCTCTAGC 360

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GCTCCCTAA 429

(2) INFORMATION FOR SEQ ID NO:9:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGCCCAAGC TTCCCATTGC ATACGTTGTA TC 32

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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(2) INFORMATION FOR SEQ ID NO:11:

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGACCTGGG AAGCCTTGGC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGGGATCCAG TCTAGAGGAT GGTCCACC 28

CLAIMS

What is claimed is:

1. A stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus, wherein the packaging cell line comprises one or more non-retroviral expression constructs which direct expression of:
   a) retroviral *gagpol* genes which produce a retroviral core virion; and
   b) a gene which encodes a pseudotyped envelope for the retroviral core virion and is under control of an inducible operator system,

wherein the protein of b) provides a pseudotyped envelope protein for the retroviral core virion resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus.

2. A stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range, wherein the packaging cell line comprises one or more non-retroviral expression constructs which direct expression of:
   a) retroviral *gagpol* genes which produce a retroviral core virion; and
   b) Vesicular Stomatitis Virus G glycoprotein under control of an inducible operator system,

wherein the Vesicular Stomatitis Virus G glycoprotein provides a pseudotyped envelope protein for the retroviral core virion resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range.

3. The packaging cell line of Claim 2 wherein the non-retroviral expression construct comprises a human cytomegalovirus immediate early promoter.

4. The packaging cell line of Claim 2 wherein the inducible operator system for expression of the Vesicular Stomatitis Virus G glycoprotein is a tet operator system.

5. The packaging cell line of Claim 2 wherein the retroviral *gagpol* genes are mutated.

6. The packaging cell line of Claim 2 wherein the cells are H29 gagpol cells.

7. The packaging cell line of Claim 5 wherein the cells are H29 new gagpol cells.

8. A stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range, wherein the packaging cell line comprises one or more cytomegalovirus expression constructs which direct expression of:

a) retroviral *gagpol* genes which produce a retroviral core virion; and

b) Vesicular Stomatitis Virus G glycoprotein under control of an inducible *tet* operator system,

wherein the Vesicular Stomatitis Virus G glycoprotein provides a pseudotyped envelope protein for the retroviral core virion resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range.

9. The packaging cell line of Claim 8 wherein the cells are H29 gagpol cells.

10. A stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range, wherein the packaging cell line comprises one or more cytomegalovirus expression constructs which direct expression of:

a) mutated retroviral *gagpol* genes; and

b) Vesicular Stomatitis Virus G glycoprotein under control of an inducible *tet* operator system,

wherein the Vesicular Stomatitis Virus G glycoprotein provides a pseudotyped envelope protein which interacts with the retroviral gagpol proteins that are expressed from the mutated *gagpol* genes to generate helper-free recombinant pseudotyped retrovirus with a pantropic host range from a stable, pseudotyped retrovirus packaging cell line.

11. The packaging cell line of Claim 10 wherein the cells are H29 new gagpol cells.

12. A method of making a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus, comprising the steps of transfecting mammalian cells with one or more non-retroviral expression constructs which direct the expression of

a) retroviral *gagpol* genes which produce a retroviral core virion, and

b) a protein which provides a pseudotyped envelope for the retroviral core virion and is under control of an inducible operator system,

wherein the protein of b) provides a pseudotyped envelope protein for the retroviral core virion

resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus.

13. A method of making a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range, comprising the steps of transfecting mammalian cells with one or more non-retroviral expression constructs which direct the expression of

a) retroviral *gagpol* genes which produce a retroviral core virion, and

b) Vesicular Stomatitis Virus G glycoprotein under control of an inducible operator system,

wherein the Vesicular Stomatitis Virus G glycoprotein provides a pseudotyped envelope protein for the retroviral core virion resulting in production of a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus with a pantropic host range.

14. The method of Claim 13 wherein the non-retroviral expression construct is a cytomegalovirus construct.

15. The method of Claim 13 wherein the inducible operator system for expression of the Vesicular Stomatitis Virus G glycoprotein is a *tet* operator system.

16. The method of Claim 13 wherein the mammalian cells are H29 cells.

17. The method of Claim 13 wherein the retroviral *gagpol* genes are mutated.

18. The method of Claim 17 wherein the mutated *gagpol* genes are the new 5′ *gagpol* and the new 3′ *gagpol* nucleotide sequences.

19. A method of making a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range, comprising the steps of:

a) transfecting mammalian cells with a first non-retroviral construct which codes for the tet operator and a second non-retroviral construct which codes for the Vesicular Stomatitis Virus G glycoprotein;

b) screening the cells of a) for tetracycline-inducible VSV-G expression in which VSV-G is not detected in the presence of tetracycline and is detected in the absence of tetracycline;

c) transfecting the cells of b) with a third non-retroviral construct which codes for the retroviral gagpol proteins; and

d) screening the cells of c) for production of retroviruses

wherein the transfected cells of d) which produce retroviruses are stable, pseudotyped retrovirus packaging cells capable of generating helper-free recombinant pseudotyped retrovirus with a pantropic host range.

20. The method of Claim 19 wherein the second and third constructs are cytomegalovirus constructs.

21. The method of Claim 19 wherein the mammalian cells are H29 cells.

22. The method of Claim 19 wherein the retroviral gagpol proteins are mutated.

23. The method of Claim 19 wherein the mutated gagpol proteins are the new 5′ gag and the new 3′ pol proteins.

24. H29 cell line.

25. H29 gagpol cell line.

26. H29 new gagpol cell line.

27. pMD.

28. pMDtet.

29. pMDtet.G.

30. pMD.gagpol.

31. pMD.new gagpol.

32. A retroviral vector for producing a cDNA library for expression in mammalian cells, comprising:
    a) two retroviral LTRs;
    b) a cloning site for insertion of cDNA; and
    c) a cytomegalovirus promoter.

33. The retroviral vector of Claim 32 wherein the retroviral LTRs are Moloney murine leukemia virus LTRs.

34. The retroviral vector of Claim 33 wherein one of the LTRs is a modified Moloney murine leukemia virus LTR in which the U3 region of the Moloney murine leukemia virus LTR is replaced with the human cytomegalovirus promoter.

35. A cDNA expression library for expression in mammalian cells, the library comprising retroviral vectors which comprise:

a) two retroviral LTRs;

b) cDNA; and

c) a cytomegalovirus promoter,

wherein the cDNA is positioned between the retroviral LTRs and is operably linked to the cytomegalovirus promoter.

36. The cDNA expression library of Claim 35 wherein the retroviral LTRs are Moloney murine leukemia virus LTRs, and one of the LTRs is a modified Moloney murine leukemia virus LTR in which the U3 region of the Moloney murine leukemia virus LTR is replaced with the human cytomegalovirus promoter.

37. A method of producing a cDNA expression library in mammalian cells, comprising the steps of:

a) introducing into mammalian cells a cDNA expression library comprising retroviral vectors which comprise two retroviral LTRs, cDNA and a cytomegalovirus promoter wherein the cDNA is positioned between the retroviral LTRs and is operably linked to the cytomegalovirus promoter; and

b) maintaining mammalian cells containing the cDNA expression library, produced in a), under conditions appropriate for expression of the cDNA expression library

whereby a cDNA expression library is produced in the mammalian cells.

38. A method of Claim 37 wherein the cDNA expression library is introduced into cells using pseudotyped retroviruses produced in a packaging cell line.

39. A method of producing a cDNA expression library in mammalian cells, comprising the steps of:

a) introducing, into a packaging cell line which produces pseudotyped retroviral particles, a cDNA expression library comprising retroviral vectors which comprise two retroviral LTRs, cDNA and a cytomegalovirus promoter, wherein the cDNA is positioned between the retroviral LTRs and is operably linked to the cytomegalovirus promoter thereby producing a packaging cell line containing the expression library;

b) maintaining the packaging cell line containing the expression library under conditions appropriate for generation of pseudotyped retroviral particles containing the RNA transcribed from the cDNA expression library;

c) infecting mammalian cells with the pseudotyped retroviruses of b),

whereby a cDNA expression library is produced in the mammalian cells.

40. A method of Claim 39 wherein the packaging cell line is selected from the group consisting of: 293GPG cells, BOSC 23 cells, PA317 cells, Kat cell line, GP+E-86 cell line, GP+EAM12 cell line, and the FLY cell line.

41. A method of producing pseudotyped retroviral particles of broad host range containing RNA transcribed from a cDNA expression library, comprising the steps of:

a) producing a cDNA expression library comprising retroviral vectors which comprise:

1) two Moloney murine leukemia virus LTRs;

2) cDNA; and

3) a cytomegalovirus promoter,

wherein the cDNA is positioned between the Moloney murine leukemia virus LTRs and is operably linked to the cytomegalovirus promoter;

b) introducing the cDNA expression library into a mammalian packaging cell line which produces pseudotyped retroviruses; and

c) maintaining the mammalian packaging cell line under conditions appropriate for production of RNA transcripts from cDNA in the cDNA expression library and for generation of pseudotyped retroviral particles containing RNA transcripts.

42. A method of Claim 41 wherein the pseudotyped retroviral particles are VSV-G pseudotyped retroviral particles.

43. A pseudotyped retroviral particle of broad host range produced by the method of Claim 42.

44. Pseudotyped retroviral particles of broad host range comprising RNA transcribed from a retroviral vector for producing a cDNA library for expression in mammalian cells, wherein the retroviral vector comprises two retroviral LTRs; a cloning site for insertion of cDNA; and a cytomegalovirus promoter.

45. Pseudotyped retroviral particles of Claim 44 which are VSV-G retroviral pseudotypes.

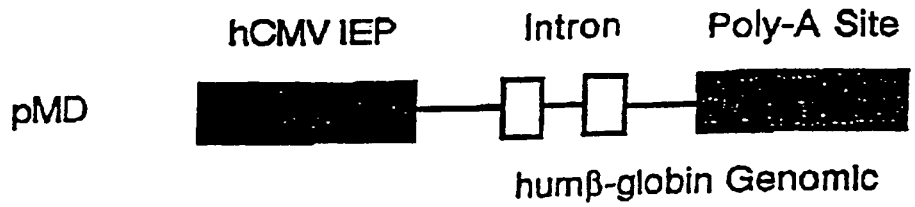


FIGURE 1

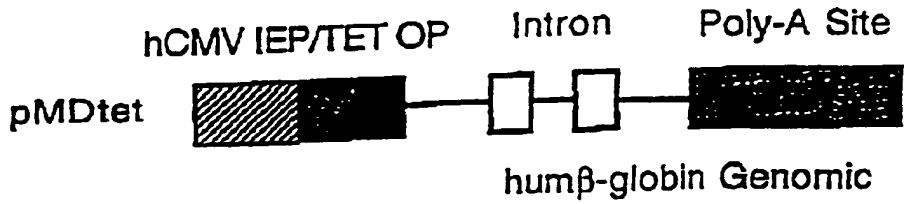

hCMV IEP/TET OP
Intron
Poly-A Site
pMDtet
humβ-globin Genomic

FIGURE 2

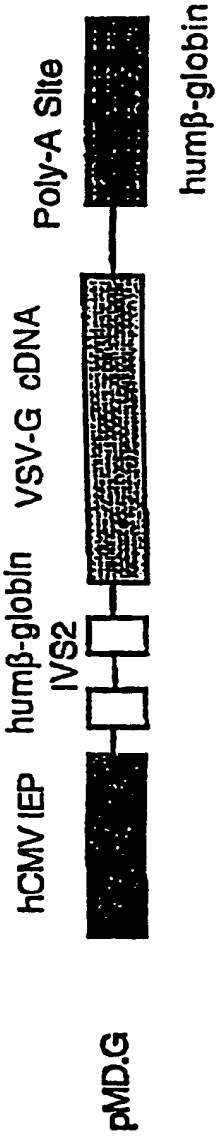
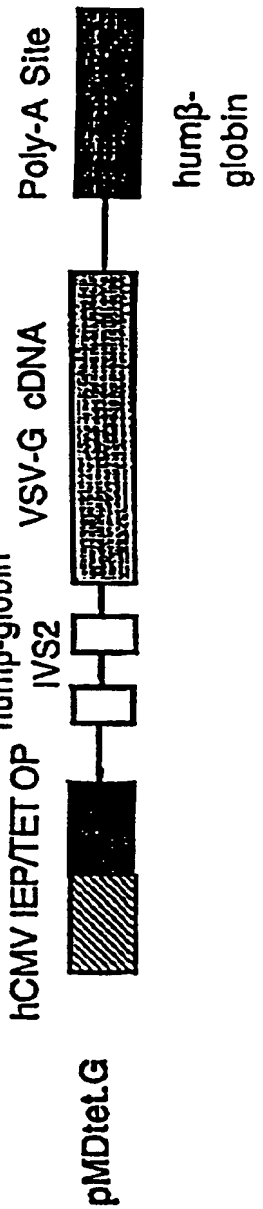

pMD.G
hCMV IEP
humβ-globln IVS2
VSV-G cDNA
Poly-A Site
humβ-globin

FIGURE 3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hCMV IEP/TET OP
humβ-globin IVS2
VSV-G cDNA
Poly-A Site
humβ-globin

FIGURE 4

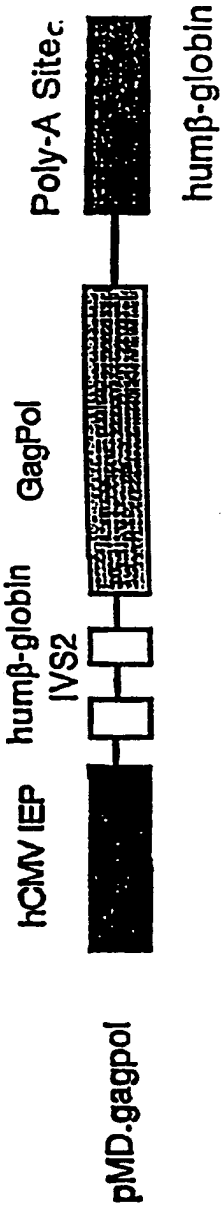

Poly-A Site
humβ-globin
GagPol
humβ-globin IVS2
hCMV IEP
pMD.gagpol

FIGURE 5

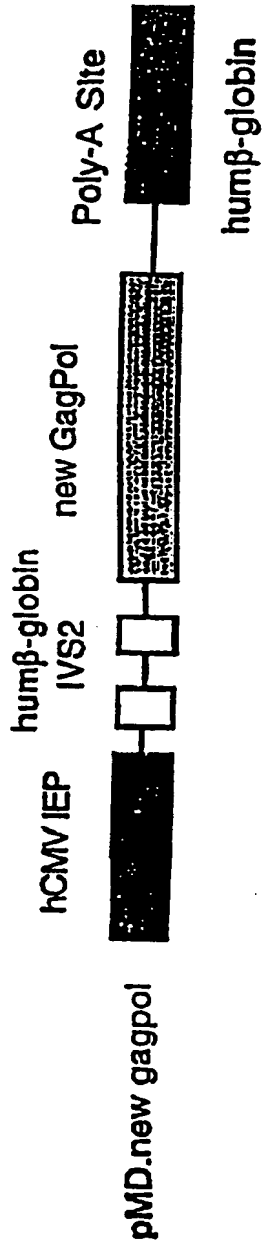

Poly-A Site
humβ-globin
new GagPol
humβ-globin
IVS2
hCMV IEP
pMD.new gagpol

FIGURE 6

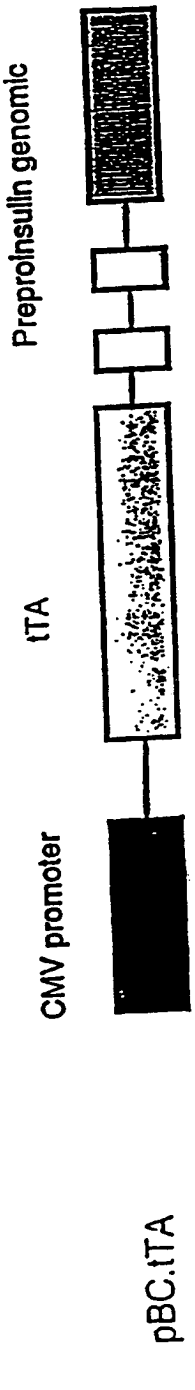


FIGURE 7

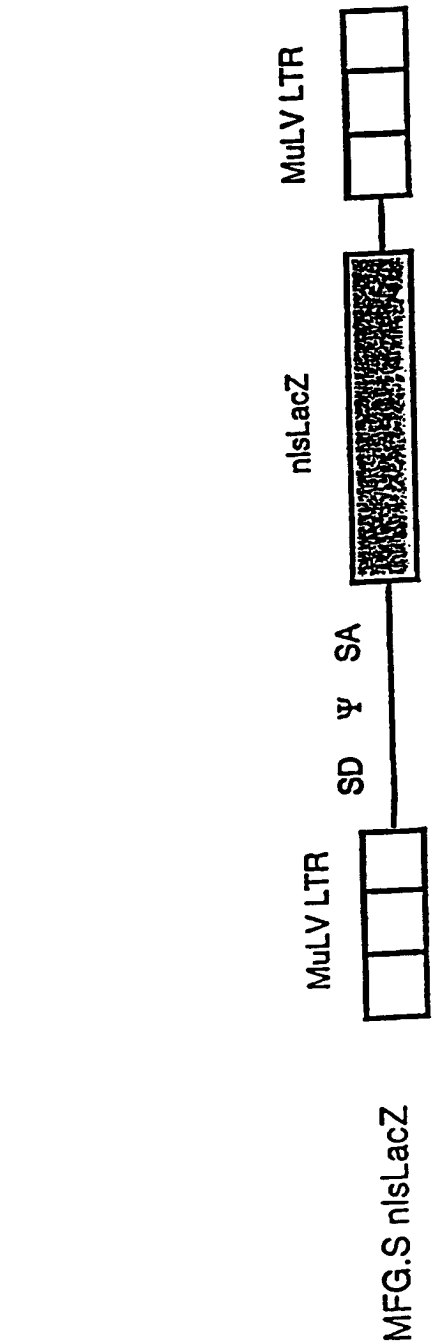


FIGURE 8

ΔU3 Retroviral Constructs

FIGURE 9A

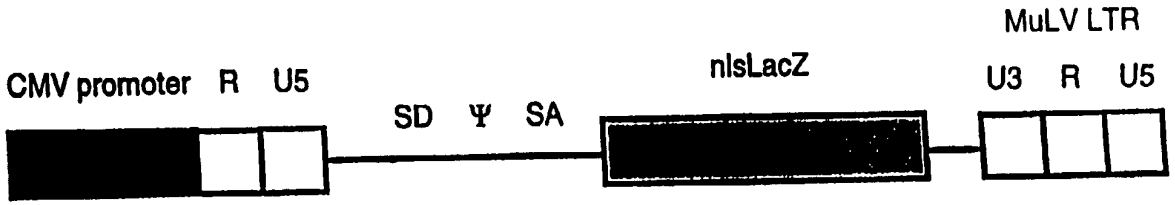
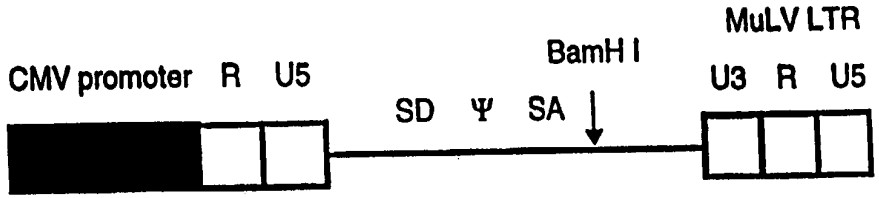


FIGURE 9B



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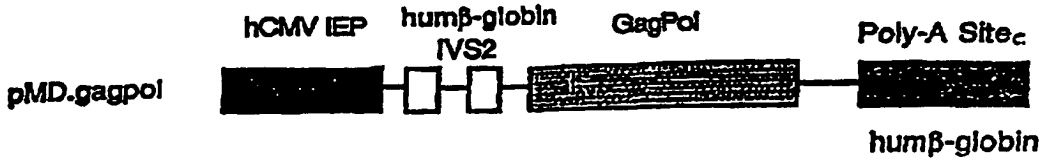

(57) Abstract

The present invention relates to a stable, pseudotyped retrovirus packaging cell line comprising packaging cells which generate helper-free recombinant pseudotyped retrovirus. The packaging cell line comprises one or more non-retroviral expression constructs, such as an expression construct with the human cytomegalovirus (CMV) immediate early promoter or derivatives of this promoter (e.g., pMD), which direct expression of: (a) retroviral *gagpol* genes and (b) a non-retroviral gene which is under the control of an inducible operator system and whose gene product pseudotypes retroviral core virions. The present invention further relates to a method of making a stable, pseudotyped retrovirus packaging cell line which generates helper-free recombinant pseudotyped retrovirus. The present invention further relates to the particular packaging cell lines described herein (i.e., H29 gagpol, H29 new gagpol) and the particular cells and constructs (i.e., packaging elements) used to produce the stable, pseudotyped retrovirus packaging cell line described herein (e.g., H29 cells and pMD, pMDtet, pMDtet.G, PMD.gagpol, pMD.new gagpol constructs). The present invention relates to a retroviral vector for producing a cDNA library for expression in mammalian cells, comprising two retroviral long terminal repeats, a cloning site for insertion of cDNA and a cytomegalovirus promoter. The invention also relates to a cDNA library for expression in mammalian cells, the library comprising retroviral vectors of the present invention. The present invention also relates to a method of expression cloning in mammalian cells. The present invention also relates to a method of cDNA expression cloning in mammalian cells. The present invention also relates to a method of identifying a gene defect responsible for a mutant phenotype using cDNA expression cloning by complementation in mammalian cells.

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INTERNATIONAL SEARCH REPORT

Intr onal Application No
PCT/US 96/17807

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUMAN GENE THERAPY, vol. 6, 1995, pages 1203-1213, XP000569741 Y. YANG ET AL.: "Inducible, high-level production of infectious murine leukemia retroviral vector particles pseudotyped with vesicular stomatitis virus G envelope protein" see the whole document, especially the abstract and introduction sections and Figures 1 and 3. ---	1-31
A	WO 94 29440 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 22 December 1994 see Examples 2, 3, 9, 11-14 and 24 and Claims. --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
13 May 1997	16.07.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Yeats, S

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 96/17807

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	METHODS CELL BIOL., vol. 43, 1994, pages 99-112, XP000570503 J.-K. YEE ET AL.: "Generation of high-titer pseudotyped retroviral vectors with very broad host range" see the whole document. ---	1-31
P,X	WO 96 04934 A (GENETIC THERAPY INC. ET AL.) 22 February 1996 see the whole document, especially Example 9 and Claims. ---	1-31
E	WO 96 35454 A (ST. JUDE CHILDREN'S RESEARCH HOSPITAL ET AL.) 14 November 1996 see the whole document. ---	1-31
P,X	PROC. NATL. ACAD. SCI. USA, vol. 93, 1996, pages 11400-11406, XP002030784 D.S. ORY ET AL.: "A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes" see the whole document. -----	1-31

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INTERNATIONAL SEARCH REPORT

rnational application No.
PCT/US 96/ 17807

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful Internatuonal Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-31

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

(a) Claims 1-31: a stable, pseudotyped retrovirus packaging cell line capable of generating helper-free recombinant pseudotyped retroviruses, wherein the packaging cell line contains one or more non-retroviral expression constructs which direct expression of retroviral gagpol genes and a gene encoding a pseudotyped envelope, e.g. the vesicular stomatitis virus G (VSV-G) glycoprotein, under the control of an inducible operator system, and methods for making the cell line.

(b) Claims 32-45: a retroviral vector for producing a cDNA library for expression in mammalian cells, comprising two retroviral LTRs, a cloning site and a cytomegalovirus promoter, the cDNA expression library per se, a method for producing the library, optioanlly by using pseudotyped retroviruses, a method of producing pseudotyped retroviral particles using the library, and the pseudotyped retroviral particles per se, which may optionally be VSV-G retroviral pseudotypes.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 96/17807

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9429440 A	22-12-94	US 5512421 A	30-04-96
		AU 7052394 A	03-01-95
		CA 2164324 A	22-12-94
		EP 0702717 A	27-03-96
		JP 8511685 T	10-12-96
WO 9604934 A	22-02-96	CA 2196208 A	22-02-96
		EP 0769968 A	02-05-97
WO 9635454 A	14-11-96	NONE	